VIROLOGY 209, 498–505 (1995)

The *Capsicum* L^3 Gene-Mediated Resistance against the Tobamoviruses Is Elicited by the Coat Protein

A. BERZAL-HERRANZ,[1] A. DE LA CRUZ, F. TENLLADO, J. R. DÍAZ-RUÍZ, L. LÓPEZ, A. I. SANZ, C. VAQUERO, M. T. SERRA, and I. GARCÍA-LUQUE[2]

Departamento de Biología de Plantas, Centro de Investigaciones Biológicas (CSIC), Velázquez 144, 28006-Madrid, Spain

Received February 9, 1995; accepted March 21, 1995

The L^3 gene is responsible for the hypersensitive response in *Capsicum* plants against infection by tobamoviruses. The resistance conferred by this gene is one of the most effective so far described against tobamoviruses. Certain isolates of pepper mild mottle virus (PMMV) are the only tobamoviruses able to overcome the L^3 resistance. Chimeric viral genomes between PMMV-S (to which L^3 plants are hypersensitive) and PMMV-I (an L^3 resistance-breaking isolate) led us to conclude that sequence variation within the coat protein gene of both isolates determines their different virulence in L^3L^3 plants. Furthermore, the results indicate that a single amino acid substitution, Asn to Met, at position 138 of the PMMV-I coat protein is sufficient to induce the hypersensitive response and localization of viral infection in *C. chinense* plants. Finally, the use of a mutant coding for a truncated coat protein (maintaining the Met_{138} coding sequence at the RNA level) demonstrates that a functional coat protein is required for elicitation of the L^3 gene-mediated resistance.

INTRODUCTION

As in many other host–virus interactions, resistance against tobamoviruses in *Capsicum* spp. is mediated by a hypersensitive reaction, manifested through the induction of necrotic local lesions. The resistance is governed by four different genes, known as L^1–L^4. They are considered to be alleles at the locus L (Boukema *et al.*, 1980; Boukema, 1982).

The tobamoviruses isolated from infected pepper plants have been classified into four subgroups of increased pathogenicity as pathotypes P_0, P_1, $P_{1,2}$, and $P_{1,2,3}$ on the basis of their interaction with the resistance genes: viruses belonging to the P_0 pathotype induce a hypersensitive reaction (HR) in the four L^1–L^4 resistance genotypes; viruses from the P_1 pathotype overcome the L^1-mediated resistance and elicit the HR in L^2, L^3, and L^4 plants; viruses from the $P_{1,2}$ pathotype infect L^1 and L^2 plants systemically, but are localized in L^3 and L^4 plants; and viruses from the $P_{1,2,3}$ pathotype infect the L^1, L^2, and L^3 plants systemically, eliciting the HR only in the L^4 plants (Boukema, 1982).

The L^3 gene-mediated resistance is one of the most effective resistance genes against tobamoviruses. Closely related isolates of pepper mild mottle virus (PMMV) are the only tobamoviruses so far described able to overcome the resistance conferred by this gene (Tóbías *et al.*, 1982; Wetter, 1984; Wetter *et al.*, 1984; García-Luque *et al.*, 1993). In contrast to most of the resistance genes against plant pathogens which segregate as dominant genes (reviewed in Fraser, 1990; Culver *et al.*, 1991; Keen, 1992), the effectiveness of the L^3 gene to localize viral infection at the inoculation sites seems to be gene-dosage dependent (Boukema *et al.*, 1980). In heterozygous plants, and under certain environmental conditions, the inoculated plants show symptoms of vein and apical necrosis, instead of necrotic local lesions.

To elucidate the viral determinants inducing the resistance conferred by the L^3 gene, we have constructed chimeric viruses between two strains of PMMV: the S strain (Alonso *et al.*, 1989) which belongs to the $P_{1,2}$ pathotype, and therefore induces a hypersensitive reaction in *C. chinense* (L^3L^3) plants, and the I strain (Wetter *et al.*, 1984) which belongs to the $P_{1,2,3}$ pathotype that systemically infects this host.

The tobamoviral genome consists of a single-stranded RNA of plus polarity of 6.5 kb. It encodes at least four proteins: 126K and 183K, involved in viral replication processes; a 30K protein, required for the cell-to-cell movement of the virus; and the coat protein (Fig. 1). The nucleotide sequences of PMMV-S and those from the coat protein gene and 3′ noncoding region of PMMV-I have been previously reported (Alonso *et al.*, 1991; García-Luque *et al.*, 1993). The 183K results from the readthrough at the amber termination codon of the 126K protein. In addition, the C-terminal region of the 183K protein might be expressed separately as a 54-kDa protein by translation of an internal open reading frame. However, at present the 54-kDa protein has not been detected in

[1] Present address: Instituto de Parasitología y Biomedicina "López Neyra" (CSIC), Ventanilla 11, 18001-Granada, Spain.

[2] To whom correspondence and reprint requests should be addressed. Fax: 34-1-562 75 18.

0042-6822/95 $6.00

plants (reviewed in Dawson and Lehto, 1990). PMMV and tomato mosaic virus Ob (ToMV-Ob) are the only tobamoviruses so far described in which none of the open reading frames overlap (Alonso *et al.*, 1991; Ikeda *et al.*, 1993; Padgett and Beachy, 1993).

The aim of this work was to identify the viral elicitors of the resistance conferred by the L^3 gene in *Capsicum* spp. plants for a better understanding of the basic mechanisms underlying the recognition events that trigger plant defense reaction.

MATERIALS AND METHODS

Virus strains, purification, and RNA extraction

The origin of PMMV-S (pathotype $P_{1,2}$) has been previously reported (Alonso *et al.*, 1989). The $P_{1,2,3}$ pathotype used in this study corresponds to an Italian isolate of PMMV (PMMV-I) which was supplied by Dr. M. Conti (Wetter *et al.*, 1984). PMMV-S and PMMV-I were initially propagated in *Nicotiana clevelandii* Gray. Virions were purified, and viral RNA was extracted from virion particles as described by Alonso *et al.* (1991) and García-Luque *et al.* (1990).

cDNA synthesis and cloning

cDNA of PMMV-I RNA was prepared as described (García-Luque *et al.*, 1993) using the oligonucleotide 5′-CTAACCTTGTCAACC (complementary to nucleotide positions 724 to 710 from the 3′-end) for priming first-strand cDNA synthesis. One of the cDNA clones termed p3P9 (see Fig. 1) was chosen for further analysis. DNA sequences were determined using specific primers or subclones generated by restriction digestion. Sequences were analyzed using the DNASTAR computer program (DNASTAR, Inc., UK).

Construction of the full-length PMMV-S cDNA transcription clone pTS

Several overlapping cDNA clones, pEC-8, p75, p85, and p4 (Alonso *et al.*, 1991), as well as the intermediate clone p54-5 (spanning from nucleotide 2149 to 5685) (F. Tenllado, I. García-Luque, M. T. Serra, and J. R. Díaz-Ruiz, unpublished data), were used to construct the pTS plasmid.

The 5′-end of the viral genome was synthesized with an oligonucleotide complementary to nucleotide 466–481 of PMMV-S (Alonso *et al.*, 1991) as primer for the first-strand cDNA and with Moloney murine leukemia virus reverse transcriptase (BRL) under the conditions described by the manufacturer. Second-strand cDNA was primed by an oligonucleotide (5′-CG**GGATCC**ATAATACGACTCACTATAGTAAATTTTTCAC) corresponding to the 13 nt at the 5′-end of the viral genome plus a T7 RNA polymerase promoter (underlined) and a *Bam*HI recognition site (bold) and synthesized by DNA polymerase. The cDNA was treated with T4 DNA polymerase and restricted with *Bam*HI. The resultant 504-bp fragment was cloned into the *Bam*HI and *Sma*I sites of pUC18. After corroborating the nucleotide sequences, the *Bam*HI–*Eco*RV fragment (413 bp) of one of the clones obtained (pT1) was ligated to the *Bam*HI–*Eco*RV-digested plasmid pEC-8 (spanning from nucleotide 34 to 2470 in PMMV-S RNA), producing plasmid pTE9. The *Sac*I fragment (nucleotide 2463–3823) from plasmid p75 (nucleotide 1960–3823) was cloned into the *Sac*I site of pTE9 to create pT126.

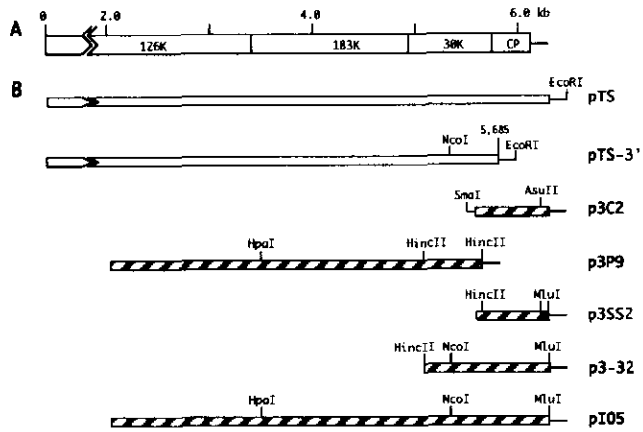

FIG. 1. (A) Genomic organization of tobamoviruses. Scheme of the coding regions for the four known proteins expressed by PMMV. (B) Construction of plasmids expressing the PMMV genome. A schematic representation of the different cDNA clones used to construct plasmid pTS containing the full-length PMMV-S genome and plasmids encoding the hybrid genomes PMMV-S/PMMV-I used in this study. Empty boxes indicate PMMV-S sequences. PMMV-I sequences are indicated by stippling. Details of these constructions are given under Materials and Methods.

Plasmid pTS-3′ (Fig. 1) was obtained after cloning the *Hpa*I–*Eco*RI fragment (nucleotide 3456–5685) from plasmid p54-5 into the corresponding sites of pT126. To allow linearization of the plasmid prior to transcription, an *Mlu*I restriction site was added just downstream of the 3′-end of the sequence in the intermediate clone p85-4. This clone was created by inserting the *Hind*III–*Bsm*I fragment (nucleotide 5187–5443) from plasmid p85 into the corresponding sites of p4 (extending from nucleotide 5221 to the 3′-end of the PMMV-S). Two oligonucleotides, 5′-ACCGCGGGTAGCGGCCCACGCGT, corresponding to the 18 nt at the 3′-end of the viral genome containing an *Mlu*I restriction site (underlined), and its complementary oligonucleotide 5′-ACGCGTGGGCCGCTACCCGCGGA, were annealed, restricted with *Sac*II, and cloned into *Sac*II–*Sma*I-digested p85-4. The *Nco*I–*Eco*RI fragment of the resultant plasmid pSS-2 was cloned into the *Nco*I–*Eco*RI-digested plasmid pTS-3′, to obtain pTS (Fig. 1).

Construction of hybrid viral genomes

Mutant and chimeric genomes were constructed at the DNA level by manipulating and cloning gel-purified DNA

restriction fragments isolated from cDNA clones. Recombinants were tested by restriction enzyme analysis and partial DNA sequencing. The resulting recombinant DNAs were used as templates for transcription by T7 RNA polymerase.

Two PMMV-I cDNA clones, p3C2, containing nucleotide sequences from nucleotide position 5576 to 6334 (García-Luque *et al.*, 1993), and p3P9 (nucleotide 2100–5622), were used (Fig. 1). The numbering is with reference to the PMMV-S nucleotide sequence (Alonso *et al.*, 1991).

First, the *Sma*I–*Asu*II fragment from plasmid p3C2 (nucleotide 5576–6325) was cloned into the *Hin*dIII-blunt-*Asu*II sites of plasmid pSS-2 to create p3SS2. Next, the 598-bp *Hin*cII fragment from plasmid p3P9 (nucleotide 5025–5622) was ligated to *Hin*cII-digested p3SS2, thus obtaining the plasmid p3-32. Plasmid pI05 was created after introducing the *Nco*I–*Kpn*I fragment from p3-32 into the *Nco*I–*Kpn*I sites of plasmid p3P9. Details of the construct are shown in Fig. 1.

DNA templates for different hybrid genomes were constructed by substituting the PMMV-S sequences contained in plasmid pTS with the corresponding sequences of PMMV-I from plasmid pI05. Hybrid genome THI-1 was constructed after introducing the 3479-bp *Bam*HI–*Hpa*I fragment from plasmid p126 (PMMV-S) into the *Bam*HI–*Hpa*I-digested plasmid pI05 (PMMV-I; see Fig. 1). pTHI-2 was created after replacing the *Hpa*I–*Nco*I fragment (nucleotide 3457–5348) from pTS (PMMV-S) with the corresponding sequence of pI05. Template pTHI-3 was produced by cloning the *Nco*I–*Eco*RI fragment from plasmid p3-32 (PMMV-I) into the *Nco*I–*Eco*RI sites of plasmid pTS-3′ (PMMV-S; see Fig. 1). Templates pTHI-4 and pTHI-5 were constructed by replacing the fragments *Nco*I–*Bsp*HI (nucleotide 5349–6070) and *Bsp*HI–*Eco*RI (nucleotide 6071–6357) from pTS with the corresponding sequences of pI05. pUC18 plasmid was used as intermediate for constructing both hybrid templates. DNA templates pTHI-6, -7, and -8 were constructed as described above but using the ATG- and TGA ATG-containing fragments.

Asn to Met_{138} substitution was introduced by PCR as described by Higuchi *et al.* (1988). Plasmid p3-32 was used as a template for mutagenic PCR. Two mutagenic DNA fragments were isolated after PCR amplification using (i) the 5′ mutagenic Met oligonucleotide (5′-GGCACGGGAATGTACAATCA) and the universal M13/pUC reverse sequencing primer 1233 (New England Biolabs) as 5′ and 3′ primers, respectively; and (ii) the universal M13/pUC sequencing primer 1224 (New England Biolabs) as the 5′ primer and the 3′ mutagenic Met oligonucleotide (5′-TTGATTGTACATTCCCGTGCC) as the 3′ primer. Both DNA fragments were denatured and renatured together and the resulting mixture was used as template for *Taq* DNA polymerase. The newly synthesized DNA duplex was PCR amplified using both universal primers 1233 and 1224. The amplified 1.4-kbp DNA fragment was digested with *Nco*I and *Eco*RI and inserted into a suitable vector for sequencing. After checking that the desired mutation had been introduced and no additional nucleotide changes had occurred within the 288-bp *Bsp*HI–*Eco*RI fragment, this fragment was purified and ligated to the *Hin*dIII–*Bsp*HI fragment corresponding to the PMMV-I (pI05) or PMMV-S (pTS) sequences for construction of pTHI-6 and pTHI-7 recombinants, respectively (Fig. 2).

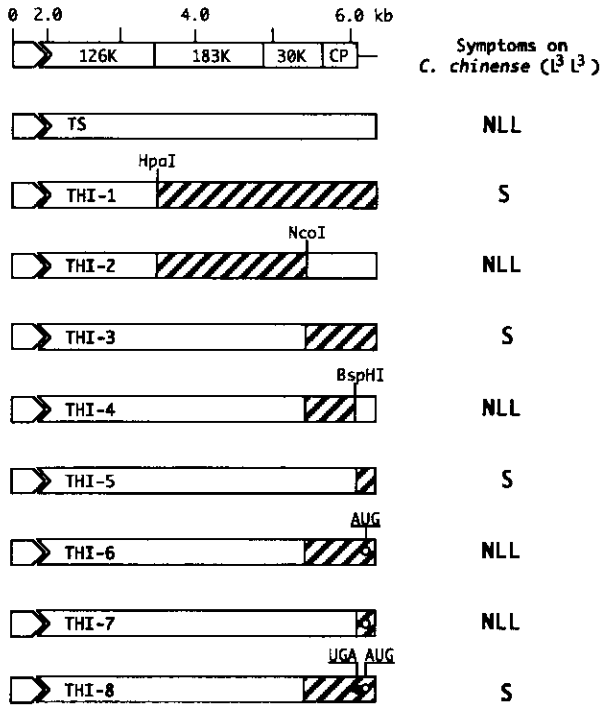

FIG. 2. Pathogenicity of different PMMV genomes on *C. chinense* plants. A schematic representation of the different hybrid viral genomes used in this study is shown on the left. A representation of the PMMV genome organization is shown above the hybrids. Symptomatology observed on *C. chinense* plants inoculated with the corresponding virus is shown on the right. NLL, necrotic local lesions, indicating localization of viral infection by the L^3 resistance gene; S, systemic infection, the viral genome is able to overcome the resistance mediated by the L^3 gene. Only important features of each hybrid genome are shown. Empty boxes correspond to PMMV-S sequences, and stippled boxes indicate PMMV-I sequences.

The sequence analysis of cloned mutagenic PCR products revealed the existence of a specific clone containing, in addition to the desired Asn to Met substitution, an extra G to T mutation 3 nt upstream of the Met codon. This mutation introduces a stop UGA codon immediately 5′ to the Met_{138} residue. We used the *Bsp*HI–*Eco*RI fragment of this mutant to construct the pTHI-8 recombinant which is identical to pTHI-6 but for the presence of the extra G to T substitution (Fig. 2).

In vitro synthesis of viral RNA genomes

Genomic RNAs were synthesized by run-off *in vitro* transcription of *Mlu*I-digested recombinants basically as

described by Beck *et al.* (1990). Reactions were stopped by addition of EDTA, pH 8.0, to 20 m*M*. An equal volume of plant inoculation buffer (50 m*M* glycine, 30 m*M* K_2HPO_4, 1% bentonite, 1% Celite) was added and the mixture was inoculated directly onto plants.

Virus propagation

In vitro-synthesized RNAs were always first inoculated onto *Nicotiana tabacum* L. Xanthi nc leaves using the inoculation buffer described above. Single necrotic local lesions were isolated from Xanthi nc tobacco leaves and homogenized in an Eppendorf tube in the presence of 20 m*M* sodium phosphate buffer, pH 7.0. The total extract was inoculated to both *C. chinense* N.J.Jacq. PI 159236 and *Nicotiana benthamiana* Domin leaves previously dusted with sterile Celite. Propagation of viral progeny from Xanthi nc tobacco plants to *C. chinense* or *N. benthamiana* plants of those viral variants coding for a truncated coat protein was done in the presence of the above bentonite-containing buffer.

Analysis of viral progenies

The viral progenies derived from the infectious transcripts were analyzed by reverse transcriptase–PCR followed by restriction enzyme digestion, as described (Tenllado *et al.*, 1994). By using the oligonucleotides 5′-CTAACCTTGTCAACC (complementary to nucleotide position 5619–5633) and 5′-GGTTCGTTTGTTTATAGA (corresponding to nucleotide 4834–4851) in RT–PCR, it was possible to amplify an 800-bp fragment. Restriction of the amplified fragment with *Eco*RI discriminates hybrid virus THI-3 from PMMV-I.

Similarly, the use of oligonucleotides CP2, complementary to nucleotide 6139–6158 of PMMV-I (5′-TTAAGGAGTTGTAGCCCACG), and CP1 (5′-GTGTACTTCTGCGTTAGG), corresponding to nucleotide 5763–5782, led to the PCR amplification of a 396-bp DNA fragment. Digestion of this fragment with *Taq*I restriction enzyme was used to identify viral RNAs as PMMV-S or PMMV-I. Finally, *Rsa*I digestion of the same fragment was used to test the presence of the Met_{138} residue-coding sequence.

The progeny viral RNA from the mutant coding for a truncated coat protein was analyzed by nucleic acid sequencing through the modified region. Nucleotide sequences were determined on RT–PCR-amplified fragments, by using the *fmol* sequencing kit (Promega). cDNA was synthesized on total leaf RNA using oligonucleotides CP1 and CP2 as primers. Total leaf RNA was extracted according to Jones *et al.* (1985).

Virus detection by ELISA

Plant extracts were prepared by homogenizing leaf tissue in phosphate-buffered saline, pH 7.4 (PBS), containing 0.2% bovine serum albumin and 0.05% Tween 20 (3 ml/g). Extracts were clarified by centrifuging at 10,000 *g* for 3 min, and the supernatants were assayed for viral coat protein by double antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977). The generation of polyclonal rabbit anti-PMMV-S serum has been previously described (Alonso *et al.*, 1991). Purified rabbit IgGs from anti-PMMV-S sera were used for plate coating at a concentration of 4 μg/ml. The IgG–alkaline phosphatase conjugates were prepared by the glutaraldehyde method (Avrameas, 1969). The ELISA reaction product was quantified colorimetrically using an EAR 400 SLT-Labinstruments microplate reader.

Protein extraction, SDS–PAGE, and Western blotting

Leaf tissue was ground in the presence of liquid nitrogen, transferred to 1.5 ml of 2× SDS–PAGE sample buffer (200 m*M* Tris–HCl, pH 6.8, 4% SDS, 200 m*M* dithiothreitol, 20% glycerol, 0.01% bromphenol blue) per gram of fresh tissue, and heated at 100° for 5 min. Total protein extracts (3 μl) were separated in the discontinuous SDS–PAGE system (Laemmli, 1970) using 4.5 and 17% stacking and resolving gels, respectively, and electrotransferred to nitrocellulose. Blots were blocked in 5% skim milk in PBS (Blotto) and incubated overnight with the anti-coat protein serum diluted 1:1000 in Blotto. After several washes with PBS containing 0.05% Tween 20, blots were incubated with peroxidase-labeled goat anti-rabbit antibodies (Sigma) diluted 1:1000 in Blotto. Enzyme was detected using 4-chloro-1-naphthol (0.2 mg/ml) as described (Harlow and Lane, 1988).

RESULTS

Generation of PMMV-S infectious transcripts

As an initial step in analyzing the induction of the hypersensitive reaction induced by PMMV-S in *C. chinense* plants, we have constructed a PMMV-S full-length cDNA clone from several overlapping cDNA clones so that infectious transcripts could be obtained. The PMMV-S genome was located downstream of a T7 RNA polymerase promoter, and an *Mlu*I restriction site was added at its 3′-end to allow linearization of the plasmid prior to transcription. The resultant plasmid pTS was transcribed with T7 RNA polymerase, and *in vitro*-synthesized capped transcripts were characterized by inoculation onto the test hosts *N. tabacum* cv Xanthi nc and *N. benthamiana*. The symptoms observed in both *Nicotiana* species were indistinguishable from those induced by the parental virus: small necrotic local lesions in Xanthi nc tobacco plants, and leaf curling and severe stunting in *N. benthamiana* plants (data not shown).

Single Xanthi nc tobacco necrotic local lesions were used to test the viral progeny derived from the infectious PMMV-S in *C. chinense* plants. The virus induced necrotic local lesions identical to the ones induced by PMMV-S. *C. chinense* plants were not directly inoculated

with the viral transcripts because the presence of bentonite in the inoculation buffer induces necrotic local lesion-like symptoms in the inoculated leaves, which can interfere with the host response against viral infection (data not shown).

Localization of the L^3 resistance-breaking determinant within the 3′-end of the viral genome

To determine whether the distinct biological properties of both PMMV-S and PMMV-I in *C. chinense* reside in the 5′ part or in the 3′ part of the viral RNA, a DNA template for the chimeric genome THI-1 containing the 5′ sequences from PMMV-S (nucleotide 1–3456) and the 3′ sequences from PMMV-I (nucleotide 3457–6356) was constructed.

The viral progeny derived from the *in vitro*-synthesized transcripts from the pTHI-1 template after a single local lesion transfer in Xanthi nc tobacco plants was inoculated onto *C. chinense* plants. At 2 weeks postinoculation, symptoms of mottling appeared in the upper noninoculated leaves (Fig. 2). The presence of virus in both the inoculated and noninoculated leaves was corroborated immunologically by ELISA, thus indicating that the ability of PMMV-I to overcome the L^3 gene-mediated resistance lies within the 3′-half of the viral genome, 3′ to the 126K protein gene.

To further localize the region involved in this phenotype, we created hybrid genomes THI-2 and THI-3. The THI-2 genome contains the region from position 3457–5348 of the PMMV-I genome substituted for the corresponding PMMV-S genome sequences, and THI-3 contains the 5′-coterminal sequences of PMMV-S (nucleotide 1–5348) and the 3′-proximal 1000 nt of PMMV-I (nucleotide 5349–6356; Fig. 2).

In *C. chinense* plants, the hybrid virus THI-2 produced necrotic local lesions identical to those induced by PMMV-S, whereas the hybrid virus THI-3 gave symptoms of mottling, similar to those showed by plants inoculated with PMMV-I. ELISA analysis of the inoculated and upper noninoculated leaves of *C. chinense* plants showed that both viruses were detected in the inoculated leaves, whereas in the upper leaves virus could be only detected in those plants inoculated with the THI-3 virus (data not shown).

The identity of the THI-3 virion RNAs purified from the systemically infected leaves of *C. chinense* plants was confirmed by PCR amplification and restriction enzyme digestion of two amplified DNA fragments: one encompassing the hybrid junction (between nucleotide positions 4834 and 5633) and the other covering part of the coat protein gene (between nucleotide positions 5763 and 6158). Therefore, the resistance-breaking phenotype maps to the 3′-proximal 1000 nt of the genome. This region contains the 3′ part of the 30K gene, the coat protein gene, and the 3′ noncoding region.

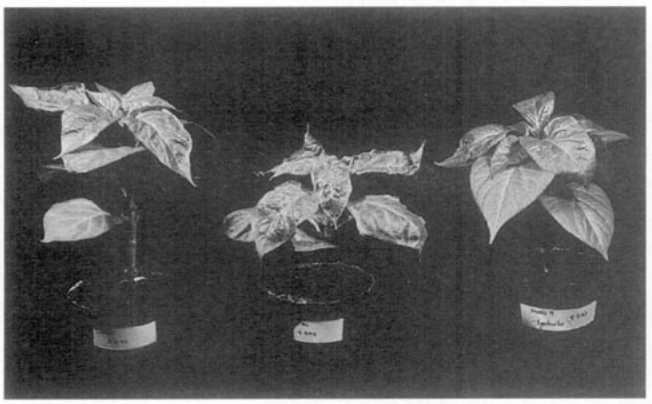

FIG. 3. Symptomatology observed on *C. chinense* plants infected with THI-5 (center) and THI-8 (right) at 2 weeks postinoculation. Plants were inoculated with a single necrotic local lesion isolated from Xanthi nc tobacco plants. Control mock-inoculated plant is shown on the left.

To precisely localize the domain(s) involved in the virus–plant interaction, we constructed two other recombinant templates, pTHI-4 and pTHI-5, coding most of the PMMV-S sequences but containing either (i) the C-terminal part of the movement protein and the coat protein N-terminal domain (nucleotide 5349–6070) (viral genome THI-4), or (ii) the coat protein C-terminal domain coding sequences plus the 3′ noncoding region (nucleotide 6071–6357) (viral genome THI-5) of the L^3 resistance-breaking PMMV-I strain (see Fig. 2).

In vitro-synthesized RNAs of both recombinants induce necrotic local lesions in Xanthi nc tobacco leaves, and no differences were observed in the symptoms induced by both variants (data not shown). However, when single necrotic local lesions from Xanthi nc tobacco leaves were propagated to *C. chinense* plants (L^3 plants), only plants inoculated with hybrid virus THI-4 showed the hypersensitive response. This hybrid resembled the pathogenicity of the parental PMMV-S ($P_{1,2}$ pathotype; Fig. 2). In contrast, plants inoculated with the viral progeny corresponding to hybrid THI-5 reproduced the symptomatology observed in plants infected with the PMMV-I strain (Figs. 2 and 3).

Immunological analysis at 15 days postinoculation showed that hybrid THI-5 was able to move from inoculated to systemic leaves, showing similar pathogenicity in pepper plants than PMMV-I (pathotype $P_{1,2,3}$; data not shown). Viral RNA progeny was isolated from purified virions obtained from systemically infected plants (*N. benthamiana* plants inoculated with hybrids THI-4 and THI-5 and *C. chinense* plants inoculated with THI-5) and the virus identity was corroborated by RT–PCR and restriction enzyme analysis (data not shown).

The results indicate that the viral domain responsible for the L^3 resistance-breaking ability of PMMV-I is encoded within the 3′-proximal 287 nt of the viral genome. These sequences correspond to the 3′-end of the coat protein gene and the 3′ noncoding region.

Coat protein Asn_{138} to Met codon substitution makes PMMV-I L^3 sensitive

There are a total of 13 nucleotide differences between PMMV-S and PMMV-I in the region 3′ to the *Bsp*HI site (Garcí-Luque *et al.,* 1993). Six of these changes are localized within the 3′-end of the coat protein gene, resulting in only 2 amino acid exchanges: Met to Asn and Ala to Val at positions 138 and 147, respectively. The Met to Asn substitution is the only nonconservative amino acid exchange observed. Therefore, to determine if the Met to Asn amino acid exchange was responsible for the different virulences between the two viral strains, we introduced a double nucleotide substitution in the PMMV-I coat protein gene, converting the Asn_{138} coding sequence into a Met codon. To analyze the effect of this mutation within the genomic background of two chimeric viruses (THI-3 and THI-5) that escaped the action of the L^3 gene, two other hybrid viruses (THI-6 and THI-7) were created (Fig. 2).

In vitro-synthesized transcripts from both constructs inoculated onto Xanthi nc tobacco leaves induced necrotic local lesions. When single necrotic local lesions were transferred to *C. chinense* plants (L^3L^3), the HR was elicited in both cases (Fig. 2). The necrotic local lesions induced by both genome variants appeared at the same time and were of sizes similar to those induced by PMMV-S (data not shown). ELISA and Western blot analysis of both inoculated and upper noninoculated leaves of infected *C. chinense* plants showed that the variants containing the PMMV-I Met coat protein gene replicated in infected leaves but they could not overcome the L^3 resistance and move to systemic leaves (data not shown).

These results reveal that the single substitution of the Asn_{138} residue coding sequence within the PMMV-I coat protein gene to a Met codon (sequence corresponding to PMMV-S), in the context of the THI-3 and THI-5 chimeric viruses, renders L^3 resistance-breaking viruses sensitive to the L^3 gene action, thus restoring the PMMV-S phenotype.

Elicitation of the hypersensitive response is a viral coat protein process

To determine whether the coat protein is required for eliciting the hypersensitive response or, alternatively, whether the RNA is sufficient for eliciting the L^3 resistance in *C. chinense* plants, we constructed a recombinant DNA template containing a stop codon UGA immediately 5′ to the Met_{138} coding sequence (pTHI-8). This recombinant exhibits the same Met coding sequence as THI-6 at the RNA level, but codes for a truncated coat protein lacking the 20 C-terminal amino acids including the Met_{138} residue.

Genomic RNA of the THI-8 hybrid was synthesized *in vitro* and inoculated onto *N. benthamiana* and Xanthi nc tobacco plants. Single necrotic local lesions were transferred from tobacco to *C. chinense* plants where the viral progeny was able to reach the upper noninoculated leaves. The mottling symptoms induced by the THI-8 genome in this host appeared at 4 weeks postinoculation, 2 weeks later than those observed in PMMV-I-infected plants (Fig. 3). However, viral RNA was detectable at 2 weeks postinoculation. The symptoms remained visible for a very short period of time and then disappeared. Nucleotide sequence of viral RNA extracted from systemically infected leaves showed that the viral progeny maintained the nucleotide substitutions introduced (data not shown).

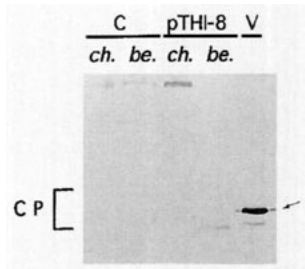

FIG. 4. Immunological detection of the truncated coat protein expressed by THI-8. Western blot analysis of total soluble proteins from systemically infected *N. benthamiana* (be.) and *C. chinense* (ch.) plants inoculated with the viral progeny corresponding to the pTHI-8 DNA template. C, healthy control plants; V, PMMV-S particles. Membranes were probed as described under Materials and Methods. Full-length coat protein is indicated by an arrow.

When total soluble protein from the systemically infected *N. benthamiana* and *C. chinense* was assayed for the coat protein by Western blot analysis, it was possible to detect a specific immunoreactive band in the infected *N. benthamiana* plants, which had a faster electrophoretic mobility than the viral coat protein, corresponding to the truncated coat protein. However, the truncated protein was not detected in the infected *C. chinense* plants (Fig. 4). This could be due either to a lower accumulation level or to a reduced stability of this protein in this host. No virion particles were detected when leaf dip preparations of the infected tissues were observed by electron microscopy (data not shown).

DISCUSSION

A mutation leading to premature termination in the coat protein gene (THI-8 hybrid virus), while not affecting the Met_{138} AUG codon sequence in the RNA, allows the L^3-sensitive THI-6 virus to systemically infect *C. chinense* plants. This finding demonstrates that the elicitation of the hypersensitive reaction in this host and the subsequent localization of the viral infection is not due to a particular feature of the RNA, but rather is due to the need for a functional coat protein. Single tobamoviral products such as the coat protein have been shown to be the unique viral factors needed for the elicitation of

hypersensitive reactions mediated by the N′ resistance gene in *Nicotiana* plants (Culver and Dawson, 1991; Pfitzner and Pfitzner, 1992). However, we cannot rule out at present that other viral factors lying in the 5′ region of PMMV-S acting in concert with the coat protein are required for inducing the L^3 gene-mediated defense response, as has been described for the formation of necrotic local lesions in *Gomphrena globosa* plants by potato virus X (Goulden and Baulcombe, 1993). The expression in L^3 plants of just those coat proteins from hypersensitive-inducing strains will elucidate this open question.

The ability of the truncated coat protein mutant to systemically infect *C. chinense* plants also indicates that the breakage of L^3 gene-mediated resistance is not due to the suppression of preexisting active host defense mechanisms by the PMMV-I coat protein. Instead, the expression of the resistance conferred by the L^3 gene is activated by the coat protein of PMMV-S. Thus, as in most plant–pathogen hypersensitive defense reactions, the specific interaction between the so-called avirulence factor from the pathogen and the product of the plant resistance gene will trigger the host defense response (reviewed in Fraser, 1990; Culver *et al.*, 1991; Keen, 1992).

Furthermore, a single amino acid substitution at position 138, converting an Asn into a Met residue in the THI-3 and THI-5 viruses' coat proteins, fully restores in the resultant hybrid viruses THI-6 and THI-7 the same ability to be localized in *C. chinense* plants as is in PMMV-S: necrotic local lesions were of the same size, and they appeared at the same rate in the three viruses (data not shown).

Based on the three-dimensional model of tobacco mosaic virus (TMV) coat protein (CP), this amino acid residue is located in an exposed position in the coat protein, lying in a β turn, connecting two α helices (Bloomer *et al.*, 1978). Therefore, it might be possible that the Met at position 138 is directly involved in the interaction with the host resistance factor. In this case, the specificity of the recognition event in *C. chinense* plants would be amino acid sequence independent, since the presence of Met in an equivalent position (position 138 in the coat protein of PMMV-S) is not conserved among all the tobamoviruses known to be localized by the action of the L^3 gene: TMV, ToMV, tobacco mild green mottle virus (TMGMV), paprika mild mottle virus (PaMMV), and ToMV-Ob. Thus, TMV and ToMV have a Leu and a Ser, respectively, in this equivalent position. It is important to note that both Leu and Met are hydrophobic amino acids and Ser is also slightly hydrophobic; whereas Asn is an hydrophilic residue.

The amino acid substitution Asn_{138} to Met in the coat protein of PMMV-I induces an important local change in the hydropathy profile of the molecule without altering the local net charge of the protein (data not shown). The change of local configuration of the protein could affect the interaction with a putative host recognition factor. It might also be possible that this amino acid substitution modifies the overall configuration of the coat protein, such that it is no longer able to interact with the factors required for the elicitation of the defense response. The ability to break the resistance against tobamoviruses conferred by the N and N′ genes has been associated with structural changes in the 126–183K and coat proteins, respectively (Saito *et al.*, 1989; Culver *et al.*, 1991; Padgett and Beachy, 1993). One can envisage that recognition by the host of an ample structural configuration in the viral elicitor rather than a primary amino acid sequence would represent some advantages for the plant; it limits the number of viral strains that break the resistance and makes the host resistance more durable. In fact, *C. chinense* plants (L^3L^3) are essentially resistant to all tobamoviruses, resulting in the formation of necrotic local lesions and limiting the spread of the virus.

Culver *et al.* (1991) have defined a domain of the tobamoviral CP, termed the "elicitor region," in which amino acid changes in the TMV CP would lead to the induction of the resistance response in N′ tobacco plants. More recently these authors have shown that the effect of those amino acid substitutions is due to a destabilization of the highly ordered structure of the TMV coat protein, leading to its recognition by the host (Culver *et al.*, 1994). The amino acid exchange reported here, converting a *Capsicum* L^3 gene resistance-breaking PMMV virus into a HR-inducing one, lies in the same "elicitor region." Although this localization may be coincidental, its effect may also be explained in the context of such model. Thus, the Asn residue might play a role in stabilizing the quaternary structure of the coat protein, preventing its recognition by the host cell and therefore leading to the HR elicitation. In addition, the localization of the identified Asn to Met amino acid substitution in the so-called elicitor region might indicate the importance of this particular coat protein region in the establishment of host–virus interactions, which in turn, and well in accordance with other plant–pathogen systems (Whalen *et al.*, 1988; Valent *et al.*, 1991; Dangl *et al.*, 1992; Goulden and Baulcombe, 1993), might be indicative of the conservation of plant defense genes in different genera.

ACKNOWLEDGMENTS

We thank J. Guijarro for photographic work, M. García de Lacoba for protein structure computer analysis, and S. Peñalver for technical assistance. This work was supported by grants from the Fundación Ramón Areces, CICYT (AGR91-0432), and the EC (BRIDGE Program BIOCT90-0156).

REFERENCES

Alonso, E., García-Luque, I., Avila-Rincón, M. J., Wicke, B., Serra, M. T., and Díaz-Ruiz, J. R. (1989). A tobamovirus causing heavy losses in protected pepper crops in Spain. *J. Phytopathol.* **125,** 67–76.

Alonso, E., García-Luque, I., de la Cruz, A., Wicke, B., Avila-Rincón,

M. J., Serra, M. T., Castresana, C., and Díaz-Ruíz, J. R. (1991). Nucleotide sequence of the genomic RNA of pepper mild mottle virus, a resistance-breaking tobamovirus in pepper. *J. Gen. Virol.* **72,** 2875–2884.

Avrameas, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. *Immunochemistry* **6,** 43–53.

Beck, D. L., Forster, R. L. S., Bevan, M. W., Boxen, K. A., and Lowe, S. C. (1990). Infectious transcripts and nucleotide sequence of cloned cDNA of the potexvirus white clover mosaic virus. *Virology* **177,** 152–158.

Bloomer, A. C., Champness, J. N., Bricogne, C., Staden, R., and Klug, A. (1978). Protein disk of tobacco mosaic virus at 2.8 Å resolution showing the interactions within and between subunits. *Nature* **276,** 362–368.

Boukema, I. W. (1982). Resistance to TMV in *Capsicum chacoense* Hunz. is governed by an allele of the L-locus. *Capsicum Newsl.* **3,** 47–48.

Boukema, I. W., Jansen, K., and Hofman, K. (1980). Strains of TMV and genes for resistance in *Capsicum.* Proc. 4th *Capsicum eucarpia* Meeting, 44–48. Wageningen, the Netherlands.

Clark, M. F., and Adams, A. N. (1977). Characteristics of a microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* **34,** 475–483.

Culver, J. N., and Dawson, W. O. (1991). Tobacco mosaic virus elicitor coat protein genes produce a hypersensitive phenotype in transgenic *Nicotiana sylvestris* plants. *Mol. Plant-Microbe Interact.* **4,** 458–463.

Culver, J. N., Lindbeck, A. G. C., and Dawson, W. O. (1991). Virus–host interactions: Induction of chlorotic and necrotic responses in plants by tobamoviruses. *Annu. Rev. Phytopathol.* **29,** 193–217.

Culver, J. N., Stubbs, G., and Dawson, W. O. (1994). Structure–function relationship between tobacco mosaic virus coat protein and hypersensitivity in *Nicotiana sylvestris. J. Mol. Biol.* **242,** 130–138.

Dangl, J. L., Ritter, C., Gibbon, M. J., Mur, L. A. J., Wood, J. R., Goss, S., Mansfield, J., Taylor, J. D., and Vivian, A. (1992). Functional homologs of the *Arabidopsis* RPMI disease resistance gene in bean and pea. *Plant Cell* **4,** 1359–1369.

Dawson, W. O., and Lehto, K. M. (1990). Regulation of tobamovirus gene expression. *Adv. Virus Res.* **38,** 307–342.

Fraser, R. S. S. (1990). The genetics of resistance to plant viruses. *Annu. Rev. Phytopathol.* **28,** 179–200.

García-Luque, I., Serra, M. T., Alonso, E., Wicke, B., Ferrero, M. L., and Díaz-Ruiz, J. R. (1990). Characterization of a Spanish strain of pepper mild mottle virus (PMMV-S) and its relation to other tobamoviruses. *J. Phytopathol.* **129,** 1–8.

García-Luque, I., Ferrero, M. L., Rodríguez, J. M., Alonso, E., de la Cruz, A., Sanz, A. I., Vaquero, C., Serra, M. T., and Díaz-Ruiz, J. R. (1993). The nucleotide sequence of the coat protein genes and 3′ non-coding regions of two resistance-breaking tobamoviruses in pepper shows that they are different viruses. *Arch. Virol.* **131,** 75–88.

Goulden, M. G., and Baulcombe, D. C. (1993). Functionally homologous host components recognize potato virus X in *Gomphrena globosa* and potato. *Plant Cell* **5,** 921–930.

Harlow, E., and Lane, D. (1988). "Antibodies, A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Higuchi, R., Krummel, B., and Saiki, R. K. (1988). A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. *Nucleic Acids Res.* **16,** 7351–7367.

Ikeda, R., Watanabe, E., Watanabe, Y., and Okada, Y. (1993). Nucleotide sequence of tobamovirus Ob which can spread systemically in N gene tobacco. *J. Gen. Virol.* **74,** 1939–1944.

Jones, J. D. G., Dunsmuir, P., and Bedbrook, J. (1985). High level expression of introduced chimeric genes in regenerated transformed plants. *EMBO J.* **4,** 2411–2418.

Keen, N. T. (1992). The molecular biology of disease resistance. *Plant Mol. Biol.* **19,** 109–122.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Padgett, H. S., and Beachy, R. N. (1993). Analysis of a tobacco mosaic virus strain capable of overcoming N gene-mediated resistance. *Plant Cell* **5,** 577–586.

Pfitzner, U. M., and Pfitzner, A. J. P. (1992). Expression of a viral avirulence gene in transgenic plants is sufficient to induce the hypersensitive reaction. *Mol. Plant-Microbe Interact.* **5,** 318–321.

Saito, T., Yamanaka, K., Watanabe, Y., Takamatsu, N., Meshi, T., and Okada, Y. (1989). Mutational analysis of the coat protein gene of tobacco mosaic virus in relation to hypersensitive response in tobacco plants with the N′ gene. *Virology* **173,** 11–20.

Tenllado, F., García-Luque, I., Serra, M. T., and Díaz-Ruíz, J. R. (1994). Rapid detection and differentiation of tobamoviruses infecting L-resistant genotypes of pepper by RT–PCR and restriction analysis. *J. Virol. Methods* **47,** 165–174.

Tóbiás, I., Rast, A. T. B., and Maat, D. Z. (1982). Tobamoviruses of pepper, eggplant and tobacco: Comparative host reactions and serological relationships. *Neth. J. Plant Pathol.* **88,** 257–268.

Valent, B., Farrall, L., and Chumley, F. G. (1991). *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* **127,** 87–101.

Wetter, C. (1984). Serological identification of four tobamoviruses infecting pepper. *Plant Dis.* **68,** 597–599.

Wetter, C., Conti, M., Altschuh, D., Tabillion, R., and Van Regenmortel, M. H. V. (1984). Pepper mild mottle virus, a tobamovirus infecting pepper cultivars in Sicily. *Phytopathology* **74,** 405–410.

Whalent, M. C., Stall, R. E., and Staskawicz, B. J. (1988). Characterization of a gene from a tomato pathogen determining hypersensitive reaction in non-host species and genetic analysis of this resistance in bean. *Proc. Natl. Acad. Sci. USA* **85,** 6743–6747.